

EXHIBIT E

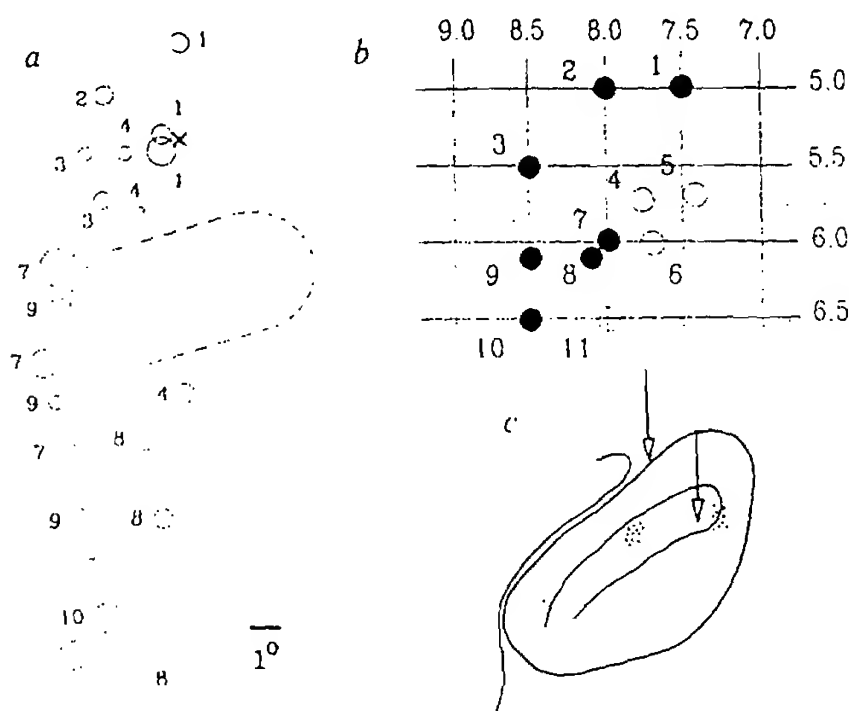


FIG. 4 Mapping of cat lateral geniculate nucleus in the same animal showing fill-in of the cortical scotoma illustrated in Fig. 3. At the two-month time point, a 1-mm wide silent area remained in the LGN. *a*, Receptive field maps of cells encountered in multiple penetrations shown in *b*. In penetration 4, there was a regular shift in receptive field position up to the edge of the scotoma, followed by a 1-mm-long zone of silence, followed by a resumption of activity on the other side of the scotoma. None of the characteristics seen in the recovered cortex, such as an overrepresentation of the perilesion retina, enlarged fields at the edge of the scotoma or bipartite fields, was seen in the LGN. *b*, Positions of penetrations in the LGN in Horsley-Clarke coordinates (mm). Several of the penetrations, covering an area ~1 mm wide, encountered visually unresponsive cells within the geniculate (open circles). Surrounding this area the electrodes encountered visually responsive cells (closed circles). *c*, Coronal view of a section through the LGN. Injections of red and green fluorescent latex microspheres (Lumafuor) were made on either side of the original cortical scotoma after the two-month survival to label the expected boundaries of the scotoma in the LGN (clusters of dots). The mapping and histology were done two weeks after the injections. Penetrations between the clusters at this antero-posterior level (open arrows) encountered visually unresponsive cells, demonstrating a sizable retained geniculate scotoma at a time when all parts of the cortex could be activated by visual stimuli. The lack of overlap between the clusters suggests further that the filling in seen in cortex is not likely to be mediated by the spread of geniculate afferents within the cortex.

response properties of cortical cells²¹. Our results suggest that the subthreshold influences of horizontal connections can be potentiated to be capable of activating the cell. The magnitude of the topographical shifts, roughly 4 to 5 mm, are large relative to the lateral spread of thalamic afferents, but the horizontal connections have an extent sufficient to account for such a reorganization. Furthermore, they are specific in connection columns of similar orientation specificity²²⁻²⁴, and could explain the orientation selectivity observed in the reorganized cortex. The receptive fields seen in the ipsilateral visual field are likely to come from the contralateral hemisphere by way of the corpus callosum.

Perhaps even more surprising than the reorganization seen in the long term are the short-term changes in receptive field size and topography. Stimulus-dependent changes in receptive field size have been observed in the somatosensory cortex, where repeated stroking of a body part over a period of months leads to a decrease of receptive field size¹¹, in effect the opposite of the type of experiment done here. Our results suggest that the subthreshold influences from outside the classical receptive field can be quickly unmasked when the ascending input is inactivated, and these short term effects represent a surprising degree of plasticity of cortical topography and receptive field structure for adult animals. The immediate changes may represent the neural substrate of perceptual phenomena such as surface fill-in

effects^{31,32}, and raises the possibility that dynamic changes receptive field structure may occur continuously during normal vision.

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Genetic immunization is a simple method for eliciting an immune response

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To produce an immune reaction against a foreign protein usually requires purification of that protein, which is then injected into an animal. The isolation of enough pure protein is time-consuming and sometimes difficult. Here we report that such a response can also be elicited by introducing the gene encoding a protein directly into the skin of mice. This is achieved using a hand-held form of the biolistic system¹⁻⁴ which can propel DNA-coated gold microprojectiles directly into cells in the living animal^{3,5,6}. Genetic immunization may be time- and labour-saving in producing antibodies and may offer a unique method for vaccination.

Young (8-15 weeks old) mice were inoculated in the ear with microprojectiles coated with plasmids containing the genomic copy of the human growth hormone (hGH) gene under the transcriptional control of either the human β -actin promoter⁷ or the cytomegalovirus (CMV) promoter⁸. Production of antibody directed against hGH was monitored by assaying sera from tail-bleeds for the capacity to immunoprecipitate ¹²⁵I-labelled hGH. Figure 1 depicts the time-course of appearance

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TABLE 1 Number of genetically inoculated mice producing antibodies

Strain	Sex	Number of mice producing anti-hGH			Number of mice producing anti-hAAT	Number of mice producing both anti-hGH and anti-hAAT
		High* titre	Low† titre	None‡		
ICR	F	18/34	12/34	4/34	2/2	4/4
CFW	M	0/4	1/4	3/4	-	-
C57BL/6	F	2/2	0/2	0/2	-	-

Antibody responses to hGH after genetic inoculation of skin cells were determined by radioimmunoassay and/or western blot analysis as described in Figs 1 and 2a. Antibodies to hAAT were determined by western blot analysis as shown in Fig. 2b. Antibodies were detectable in most mice within 2 weeks after the primary inoculation. The four ICR mice and the three CFW mice that failed to respond to genetic immunization were killed about 1 month and 2 months after the primary inoculation, respectively. The sources of mice were: ICR (Harlan), CFW (Charles River) and C57BL/6 (Jackson).
 *High titre, >0.5 ng hGH precipitable per μ l of serum about 1 month after the primary inoculation.
 †Low titre, 0.02-0.5 ng hGH precipitable.
 ‡None, <0.02 ng hGH precipitable.

anti-hGH antibody in three individual mice representing high, medium and low responses. Table 1 shows that 88% (30/34) of ICR strain mice produced antibodies within a few weeks of inoculation. The CFW strain male mice seem less responsive to this approach than the ICR and C57BL/6 strain female mice tested. The antibody titres of mice showing responses to the hGH gene were arbitrarily grouped into high and low categories. Fifteen out of 34 ICR mice had antibodies capable of precipitating >0.5 ng hGH protein per microlitre of serum about one month after the primary inoculation. Nanograms of hGH protein could be detected in the genetically inoculated skin one day after inoculation⁵, but not in the blood of the treated mice.

Several lines of evidence support the conclusion that antibody is being produced against hGH. First, preimmune sera (Fig. 1) and sera from control mice inoculated with irrelevant plasmids (data not shown) precipitated background amounts of hGH. Second, the precipitation of labelled-hGH by the test sera was specifically competed by purified, unlabelled-hGH (data not

shown). Third, a 1:5,000 dilution of the test sera reacted in a western blot with purified hGH but not with other proteins (Fig. 2a).

We used the following protocol to investigate whether the primary response could be augmented. Three mice that had received a primary genetic inoculation and were producing anti-hGH antibody were inoculated again with the hGH plasmid. A fourth mouse that was also producing anti-hGH antibody was treated with a control plasmid expressing the firefly luciferase gene. There were variable but distinct responses to the hGH-DNA boost whereas the control boost showed little change in antibody levels (Fig. 3). Mice producing anti-hGH antibody showed no local inflammation when bombarded again in the ear with the hGH plasmid. We thus conclude that it may be possible to augment the immune response by subsequent DNA boosts. In addition to variations in the genetic background of individual mice, some of the variability in the level of anti-hGH produced by both primary and secondary treatments

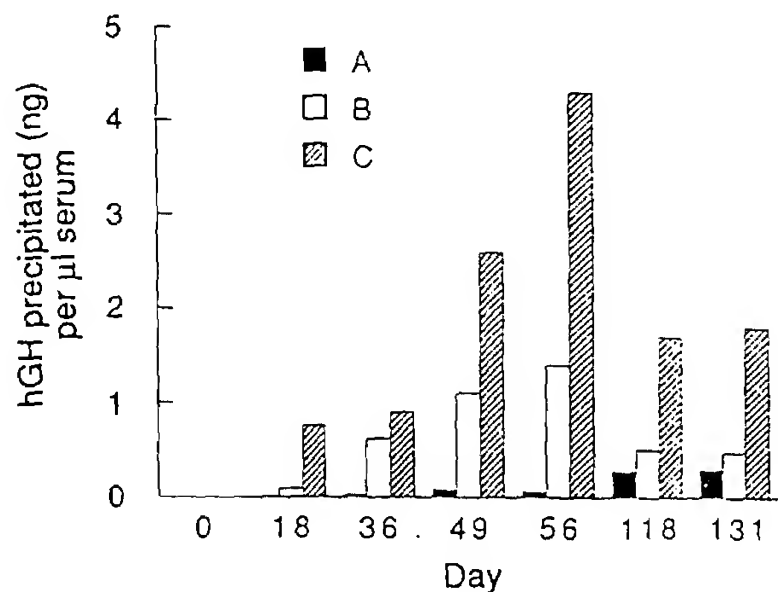


FIG. 1 Time course for the production of antibody to hGH in three mice (A, B, C) after genetic inoculation.

METHODS. Mice were inoculated in the skin of the ear with a plasmid expressing the hGH gene. Blood (50-100 μ l) was withdrawn from the tail on the days indicated. The amount of antibody to hGH, under linear assay conditions, was assayed by incubating 1 μ l diluted sera with 1 μ l ¹²⁵I-labelled hGH (DuPont-NEN, 84-88 μ Ci ml⁻¹; 113-116 μ Ci μ g⁻¹) for 1 h at room temperature. Protein A-agarose beads (4 μ l; Pierce) were added and the mixture incubated for 12-18 h at 4°C. The beads were then pelleted by centrifugation and washed thoroughly with PBS before determining the c.p.m. bound. Sera from pre-genetic immunization and from mice inoculated with a luciferase gene precipitated an average of 0.2 ng ¹²⁵I-labelled hGH. Values

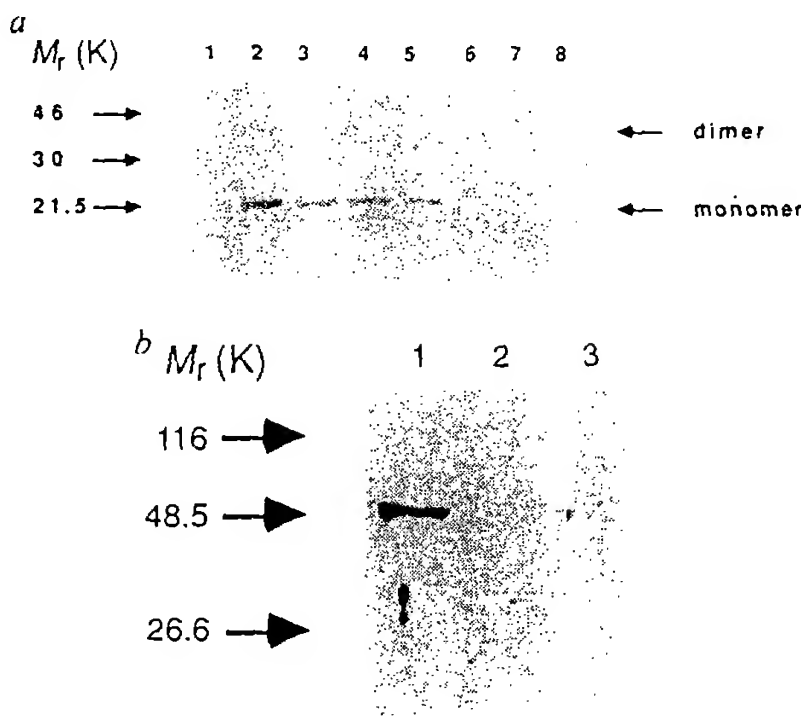


FIG. 2 a, Detection of antibodies against hGH in the sera of genetically immunized mice by western blot analysis. Serum from a genetically immunized animal (118 days after the primary inoculation) was diluted 1:5,000 and reacted with purified hGH protein (CalBiochem) that had been separated in a 12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). Lane 1, 1 μ g luciferase protein; lanes 2-7: hGH, 1 μ g (lane 2), 0.5 μ g (lane 3), 0.25 μ g (lane 4), 0.125 μ g (lane 5), 0.0625 μ g (lane 6), 0.0312 μ g (lane 7); lane 8, 1 μ g BSA. b, Detection of antibodies against hAAT. Serum from an immunized mouse (25 days after the primary inoculation) was diluted 1:5,000 and reacted with purified hAAT protein (CalBiochem) that had been separated in a 12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore). Lane 1, 1 μ g luciferase protein; lanes 2-3: hAAT, 1 μ g (lane 2), 0.5 μ g (lane 3); lane 4, 1 μ g BSA.

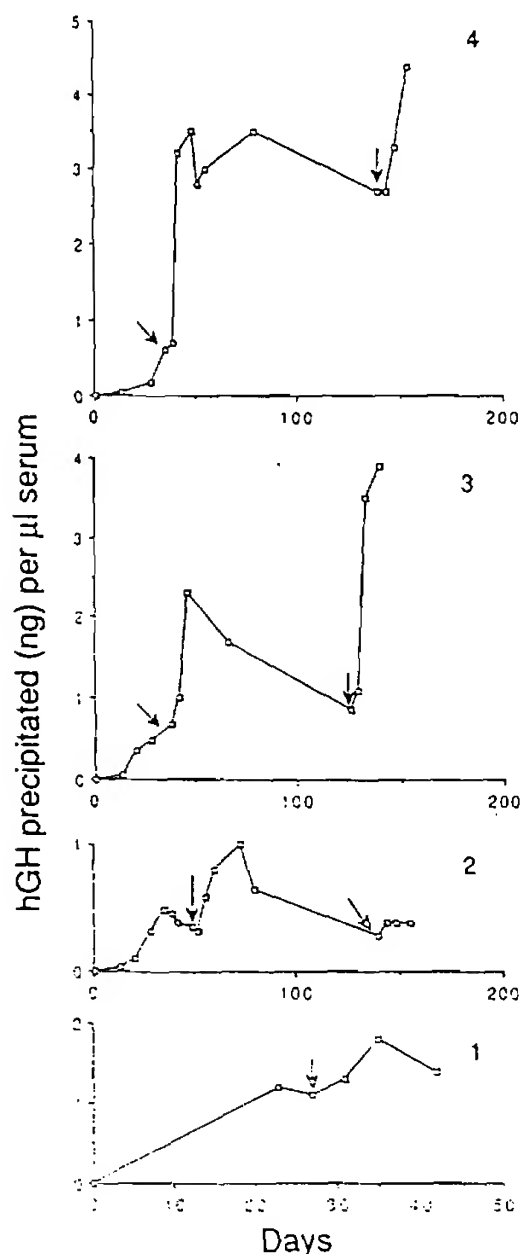


FIG. 3 Effects of genetic immunization boosts on anti-hGH antibody production. Four mice producing antibodies against hGH after a primary treatment at day 0 were inoculated again at different times with either the hGH plasmid (solid arrows) or the luciferase plasmid (open arrows). Three of the mice were inoculated a third time with either the luciferase plasmid (number 2) or the hGH plasmid (numbers 3 and 4). The sera were assayed as described in Fig. 1. The line between the first and second data points for number 1 is included for visualization.

probably arises from the operation of the device or the coating of the DNA onto the microprojectiles because treatment of skin cells with a luciferase reporter plasmid also leads to variable levels of expression^{5,6}.

We have tested whether this technique might be generally applicable to other proteins by genetically inoculating with a plasmid containing the complementary DNA copy of the human α 1-antitrypsin (hAAT) gene. This gene was also expressed from the CMV promoter and introduced into the skin of the ear. All the mice inoculated produced antibody to hAAT (6/6; Table 1) as determined by western blot analysis (Fig. 2b). Mice genetically immunized with both hAAT and hGH at the same time produced antibody to both proteins (4/4). That the two mice inoculated with only the hAAT plasmid produced anti-hAAT antibody demonstrates that the pharmaceutical effect of hGH is not required to elicit the response. Our results are from inoculations with the hand-held biolistic device. But a simple adaption⁶ of the commercially available biolistic instrument (BioRad) can also be used for genetic immunization. By contrast, injection of the hGH plasmid (50 µg) into the skin of two mice with a hypodermic needle did not produce a response. Biolistic inoculations into the liver produced hGH⁵ but did not elicit an immune reaction (eight mice tested).

produce antibodies to particular proteins by eliminating the steps for protein purification and adjuvant administration. The second, more speculative, is the genetic vaccination of animals against pathogenic infection by producing foreign antigens in restricted subsets of self-cells that mimics natural infections. In this regard, differences in immunological response between genetic and conventional immunization (for example, the duration and magnitude of antibody production or level of T-cell response) may give this protocol useful features. The ability to raise antibodies to both hGH and hAAT indicates that this technique may generally be applicable to secreted proteins. Whether it will be effective with nonsecreted proteins remains to be seen. This technique offers a simple method to elicit antibodies to some proteins and may provide a tool for manipulating the immune response. □

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Targeted disruption of μ chain membrane exon causes loss of heavy-chain allelic exclusion

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BURNET'S clonal selection theory¹ suggests that each B lymphocyte is committed to a single antibody specificity. This is achieved by a programme of somatic rearrangements of the gene segments encoding antibody variable (V) regions, in the course of B-cell development^{2,3}. Evidence from immunoglobulin-transgenic mice and immunoglobulin-gene-transfected transformed pre-B cells suggests that the membrane form of the immunoglobulin heavy (H) chain of class μ (μ m), expressed from a rearranged H-chain (IgH) locus, may signal allelic exclusion of the homologous IgH locus in the cell⁴⁻⁶ and initiation of light (L)-chain gene rearrangement in the Ig κ loci⁶. We report here that targeted disruption of the membrane exon of the μ chain indeed results in the loss of H-chain allelic exclusion. But, some κ chain gene rearrangement is still observed in the absence of μ m expression.

The μ MT mouse mutant was generated by targeted disruption of the membrane exon of the μ chain by a neomycin resistance gene in embryonic stem cells, and then introduction of the mutation into the mouse germ line⁷. Homozygous mutant mice cannot produce B cells because B-cell development is arrested at the stage of pre-B cells. But in heterozygous mutant mice normal numbers of B cells are produced. These cells express surface-bound immunoglobulin molecules whose μ chains are encoded by the wild-type IgH locus⁷. This allows a direct test of whether μ m expression is required for allelic exclusion at the H chain locus. The first gene rearrangement occurring in the course of B-cell development is the joining of D_H- and